SPECIFICATION

THERMOPHILIC ENZYMES HAVING β-GLYCOSIDASE ACTIVITY

BACKGROUND OF THE INVENTION

The present invention relates to a thermophilic enzyme having β-glycosidase activity. More particularly, the present invention relates to a thermophilic enzyme having β-glycosidase activity derived from a hyperthermophilic bacterium belonging to the genus Pyrococcus.

β-Glycosidases are useful for hydrolysis of saccharides, DNA sequencing, conformational analysis of glycoproteins and glycolipids, and enzymatic synthesis of oligosaccharides and heterosaccharides with high optical purities. The catalytic reaction of β-glycosidases with substrates is specific with respect to the types of the monosaccharides constituting the substrates, and the optical isomerism and the position of the glycosidic linkage to be cleaved in the substrates. β-Glycosidases are also useful for the modification of sugar chains and the synthesis of oligosaccharides and polysaccharides retaining their optical stereoisomerism, as well as the synthesis of heterosaccharides (e.g., biosurfactants) due to their ability to transfer a glycoside group into a primary, secondary or tertiary alcohol. Hitherto, various types of β-glycosidases with different substrate-specificities have been found in bacteria and plants. However, since many of such β-glycosidases are derived from mesophilic organisms, they are poor in thermal

resistance, and consequently are unsuitable for use in synthetic reactions under such extreme conditions that organic solvents are used simultaneously.

If a thermophilic β-glycosidase active in organic solvents is found, this can be used as an biocatalyst to develop a new procedure for synthesizing a heterosaccharide with high optical purity. In this procedure, the reverse hydrolytic reaction (i.e., synthetic reaction) is utilized which predominately occurs in the presence of an organic solvent. Under the circumstances, a novel β-glycosidase which is active under extreme conditions has been strongly demanded.

SUMMARY OF THE INVENTION

An object of the present invention is to provide a thermophilic enzyme with β-glycosidase activity.

For solving the above-mentioned problems, the present inventors focused on hyperthermophilic bacteria capable of growing within the temperature range from 90 to 100° C. As a result, they have found a gene that is assumed to encode a protein having β-glycosidase activity from its nucleotide sequence. The inventors have succeeded in the production of an enzyme from the gene by introducing the gene into Escherichia coli cells to transform the cells and then producing the enzyme from the transformants, which enzyme was confirmed to be stable at high temperatures (90° C or higher) and to have β-glycosidase activity. This success leads the accomplishment of the invention.

That is, the present invention provides a thermophilic

enzyme having β-glycosidase activity which comprises the amino acid sequence of SEQ ID NO: 2 in which one or a plurality of amino acid residues may be deleted, replaced or added. The number of the amino acid residue which may be deleted, replaced or added in the amino acid sequence of SEQ ID NO: 2 is not particularly limited as long as the β-glycosidase activity is retained, but preferably from 1 to 30, and more preferably from 1 to 18. It is preferable to delete, replace or add an amino acid residue or residues present in any of the regions of amino acid residues 78-86, 154-171 and 1-423. The enzyme preferably has an optimum temperature of 100° C or higher.

The present invention also provides a DNA which is capable of hybridizing to the nucleotide sequence of SEQ ID NO: 1 or to the complement thereof under such conditions that the hybridization is carried out in 6xSSC and 50% formamide at 42 ℃ and the washing process is carried out in 6xSSC and 40% formamide at 25 ℃, and which encodes a thermophilic enzyme having β-glycosidase activity. These conditions are of low stringent. A moderate stringent conditions are such that the hybridization is carried out in 6xSSC and 40% formamide at 42 ℃ and the washing process is carried out in 1xSSC and 0% formamide at 55 ℃. A high stringent conditions are such that the hybridization is carried out in 6xSSC and 30% formamide at 42 ℃ and the washing process is carried out in 0.1xSSC and 0% formamide at 62 ℃. The DNA may encode a thermophilic enzyme which comprises the amino acid sequence of SEQ ID NO: 2 in which of one or a plurality of amino acid residues may be deleted,

replaced or added and which has β-glycosidase activity.

The present invention further provides a recombinant vector containing the DNA therein, a host cell transformed with the recombinant vector, and a process for producing the enzyme comprising culturing a host cell transformed with an expression vector containing a DNA encoding the enzyme and then collecting the enzyme from the resultant culture. Using this process, the mass production of the enzyme becomes possible.

The present invention further provides a process for the hydrolysis of a β-glycoside having a long alkyl chain at the reducing end, with a thermophilic enzyme having β-glycosidase activity which comprises the amino acid sequence of SEQ ID NO: 2 in which one or a plurality of amino acid residues may be deleted, replaced or added. The long alkyl chain may be an alkyl group having carbon atoms of 8 or more. The hydrolysis may be carried out at a temperature of 85° C or higher, and preferably 100° C or higher.

This specification includes part or all of the contents as disclosed in the specification and/or drawings of Japanese Patent Application No. 10-222866, which is a priority document of the present application and incorporated herein by reference in its entirety.

The above and other objects, effects, features and advantages of the present invention will become more apparent from the following description of embodiments thereof taken in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the effect of the Triton X-100 concentration on the His-BGPh Activity. The standard of 100% was defined as the activity at 0.1% Triton X-100.

Figure 2 shows thermostability of His-BGPh at 90℃. Triton X-100 at 0.1% was present in the reaction mixtures. The standard of 100% was defined as the activity without heating.

Figure 3 shows optimum pH of the activity for His-BGPh. The OD405 indicates the amount of released p-Nph group in acetate buffer (square) and phosphate buffer (circle). The closed symbols correspond to the activity of BGPh and open symbols correspond to the activity of His-BGPh. For these measurements, equal amounts of BGPh and His-BGPh were used because the heated suspension I (BL21(DE3)/pET-11a/BGPh or BL21(DE3)/pET-15b/BGPh) was estimated to contain the same amount of each induced protein by quantification using SDS-PAGE analysis.

Figure 4 shows temperature dependency of BGPh. Optimum temperature was determined by the plots of enzymatic activity (OD405 nm change) against reaction temperature. An Arrhenius plot of the data is given in the inset.

Figure 5 shows aligned amino acid sequences of five β-glycosidases from hyperthermophilic archaea. The abbreviations of the sources of the enzymes are : BGPh, β-glycosidase from P. horikoshii; BMPh, a β-mannosidase gene homolog from P. horikoshii (8, 9); BGPf,

β - glucosidase from P. furiosus (17); BMPf, β - mannosidase from P. furiosus (17); Sβ - gly, β - glycosidase fromSulfolobus solfataricus (18). The conserved residues, identified automatically by the GeneWorks program, are shown in the open boxes. The reversed open triangles indicate the location of the nucleophile (E324) and the putative acid/base catalyst (E155 and H111) with R75 in the spatial proximity of the nucleophile of BGPh. The arrow shows the prominent deletion of more than 30 residues found in BGPh.

Figure 6 shows illustrated location of the four hydrophilic edges on the tetragonal structure of Sβ - gly (30) and the four hydrophobic areas exposed by removing the hydrophilic loops forming the edges. (A) The tetragonal arrengement with the hydrophilic edges (blue). (B) The tetragonal arrengement with a hydrophobic surface (red) created by the deletion of the hydrophilic loops, shielding barrel helices 3 and 4 from solvent.

Figure 7 shows a comparison of hydropacy profiles between BGPh and Sβ - gly. The panel (A) shows the hydropacy profile of BGPh and panel (B) shows the hydropacy profile of Sβ - gly. The arrows indicate the corresponding residue numbers. Two hydrophobic clusters are observed in BGPh but not in Sβ - gly.

DESCRIPTION OF THE PREFERRED EMBODIMENT

The present invention will be described specifically below.

The enzyme according to the present invention is a

thermophilic enzyme having β-glycosidase activity which comprises the amino acid sequence of SEQ ID NO: 2 in which one or a plurality of amino acid residues may be deleted, replaced or added. The enzyme comprising an amino acid sequence of SEQ ID NO: 2 and having β-glycosidase activity is derived from a sulfur-metabolizable thermophilic archaeon Pyrococcus horikoshii (the accession number: JCM 9974). One example of the processes for producing the enzyme is described below.

First, cells of Pyrococcus horikoshii are cultured and then chromosomal DNA was prepared therefrom. The chromosomal DNA is digested with restriction enzyme(s) to give fragments, and a genomic DNA library is constructed using the fragments. Clones which cover the chromosome of Pyrococcus horikoshii are selected and aligned. The aligned clones are sequenced and a gene encoding a β-glycosidase is identified. The nucleotide sequence of the gene encoding β-glycosidase is depicted in SEQ ID NO: 1. The gene is amplified by the PCR method and then extracted. The extracted gene is inserted into an expression plasmid suitable for protein production (e.g., pET11a or pET15b). The resultant recombinant plasmid is introduced into cells of a host (e.g., Escherichia coli), from which the enzyme can be produced. The produced enzyme is isolated and purified by heating and then subjecting to column chromatography.

As a result, it is revealed that the purified enzyme is a protein having a molecular weight of about 45,000 Da and capable of hydrolyzing β-glycosides. When the enzyme

is treated in 50 mM phosphate buffer (pH 6.0) containing 250 mM NaCl at 95° C for 1 hour, its activity is retained at the level of 80% based on the initial level. The enzyme has an optimum pH of pH 6.0 and an optimum temperature of 100° C or higher in terms of the enzymatic activity.

Variants of the enzyme, that is, thermophilic enzymes comprising deletion, replacement or addition of one or a plurality of amino acid residues in the amino acid sequence of SEQ ID NO: 2 and having β-glycosidase activity, may be prepared by any known techniques, such as site-specific mutagenesis and the PCR method.

The enzymes of the present invention can be used for hydrolysis of saccharides, DNA sequencing, conformational analysis of glycoproteins and glycolipids, synthesis of origosaccharides and heterosaccharides with high optical purities, and the like.

DEPOSIT OF MICROORGANISM

A transformant designated "E. coli BL21 (DE3) pET15b/Gly2M" which is E. coli BL21 (DE3) transformed with an expression vector containing a β-glycosidase gene (pET15b/Gly2M) was deposited under the terms of the Budapest Treaty on July 27, 1999 at the National Institute of Bioscience and Human-technology, Agency of Industrial Science and Technology, Japan (1-3, Higashi 1-chome, Tsukuba-shi, Ibaragi-ken, Japan) under Accession No. FERM BP-6800.

The following examples are given as more specific

illustration of the invention. It should be understood, however, that the invention is not limited to the specific details set forth in the examples.

EXAMPLES

Abbreviations: BGPh, β-glycosidase from P. horikoshii; BMPh, a β-mannosidase gene homolog from P. horikoshii; BGPf, β-glucosidase from P. furiosus; BMPf, β-mannosidase from P. furiosus; Sβ-gly, β-glycosidase fromSulfolobus solfataricus; Amp, ampicillin; IPTG, isopropyl-β-D-thiogalactopyranoside; His-BGPh, BGPh with His-tag; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; CBBR, Coomassie Brilliant Blue R; X-Glu, 5-bromo-4-chloro-3-indolyl-β-glucopyranoside; p-Nph-β-D-Glcp, p-nitrophenyl β-D-glucopyranoside; LA-β-D-Glcp, β-D-glucopyranosides with long alkyl chains.

MATERIALS AND METHODS

Chemicals - The pET-11a vector and ultracompetent E. coli XL2-Blue MRF' cell were purchased from Stratagene. The pET-15b vector and E. coli strain BL21 (DE3) were obtained from Novagen. Vent DNA polymerase was purchased from New England Biolabs. Restriction enzymes were purchased from Promega and Toyobo (Osaka, Japan), and were used according to the manufacturers' recommendations. Ultrapure deoxynucleotide solution (dNTPs) was obtained from Pharmacia Biotech. Isopropyl-β-D-thiogalactopyranoside (IPTG) was from Takara Shuzo (Otsu, Shiga, Japan).

Cloning of Genes and Construction of Expression Vector - The genome of P. horikoshii was sequenced using the method of Kaneko et al. (10). Standard cloning techniques were used throughout. The expression vectors pET-11a and pET-15b were double-digested by the restriction enzymes Nde I and BamH I and the resulting 5.7Kbp fragment was purified with a QIAquick Gel Extraction Kit (QIAGEN). The gene coding β - glycosidase (BGPh) was amplified by the PCR method using the following two primers: upper primer, TAAGAAGGAGATATACATATGCCGCTGAAATTCCCGGAAATGTTTCTCTTTGGT ACC (SEQ ID NO: 3); lower primer, TTTACTGCAGAGAGGATCCCTAATCCTAAAGTTGAAGTTCTGGTAG (SEQ ID NO: 4). The PCR product was cloned into expression vectors pET-11a and pET-15b using NdeI and BamHI sites.

The digested 1.3 Kbp fragment coding BGPh was purified and ligated to the insertion sites of the pET-11a and pET-15b vectors. Ultracompetent E. coli XL2-Blue MRF' cells were transformed with the recombinant molecule. Transformants were screened on 2 xYT plates containing 50 mg/ml of ampicillin (Amp) incubated at 37℃ overnight. The transformant colonies were propagated in 5 ml 2 x YT +Amp medium at 37℃ overnight and the vectors pET-11a/BGPh and pET-15b/BGPh were purified after centrifugation using a Mini Plasmid Kit (QIAGEN). The pET-11a/BGPh and pET-15b/BGPh were double-digested with NdeI and BamHI and the insert length was checked using agarose gel electrophoresis. The absence of additional mutations within the coding region of BGPh was verified by sequencing on an Applied Biosystems 373A DNA sequencer (Taq DyeDeoxy

Terminator Cycle Sequencing Kit, PerkinElmer).

Overexpression and Purification of Recombinant Protein - The E. coli strain BL21 (DE3) was transformed with the pET-11a/BGPh plasmid to express mature BGPh and pET-15b/BGPh plasmid to express His-tagged BGPh. The transformant colony was propagated as seed culture in 200 ml 2 x YT +Amp medium at 37℃ overnight. An inoculate of 40 ml seed culture was inoculated to 2 l of 2 x YT +Amp medium. The transformant was induced at OD600=1 with 1 mM IPTG for 4 h. The induced cells were collected by centrifugation and stored at -20℃ .

The frozen cells (7 g) were thawed and mixed with 10 ml of 50 mM Tris-HCl buffer (pH 7.5) and 5.6 ml of 10% Triton X-100, resulting in a final concentration of 2.5%. The cell suspension was heated at 85℃ for 10 min, then centrifuged at 5000 x g for 20 min. The supernatant was collected and stored at 4℃ . The cell pellet was mixed with the same volume of the buffer and Triton X-100 and heated again. The heated sample was centrifuged at 25000 x g for 20 min. The combined supernatant was mixed with 1 mg of bovine DNase 1 (Sigma) and incubated at 37℃ for 30 min. The supernatant was heated at 85℃ for 10 min, then centrifuged at 25000 x g for 20 min to remove the inactivated DNase.

The solubilized recombinant BGPh with His-tag (His-BGPh)was subjected to affinity chromatography with Ni-conjugated Sepharose, using a stepwise elution from 5 mM to 1 M imidazole in 20 mM Tris-HCl (pH 8.0) with 0.5 M NaCl solution (His-bind Buffer Kit, Novagen) containing 0.1% Triton X-100. BGPh was eluted with 100 mM imidazole

with 0.1% Triton X-100. The enzyme samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (11); a low molecular weight electrophoresis calibration kit, purchased from Pharmacia Biotech, was also run. For SDS-PAGE (PhastGel, 10-15%), the enzyme sample (5 ml) was mixed with SDS sample buffer (5 ml), boiled for 5 min, mixed with marker dye (1 ml) and applied to the gel in 1 or 4 ml aliquots. Following electrophoresis, protein was detected by Coomassie Brilliant Blue R (CBBR) staining according to the manufacturer's recommendation. The His-tagged protein was detected with QIAexpress Detection System (QIAGEN) after blotting onto a nitrocellulose membrane (Pharmacia Biotech).

Cellular Localization of the Activity - Localization of the BGPh activity in E. coli transformant cells (BL21(DE3)/pET-11a/BGPh or BL21(DE3)/pET-15b/BGPh) was examined by fractionation of the cell components. The cell membrane was isolated as follows: 7 g of the induced cells, which were frozen at -20℃ , were thawed and mixed with 10 ml of 50 mM Tris-HCl buffer (pH 7.5). The cell suspension (suspension I) was sonicated with a Sonifier 250 (Branson) for 4 min at an output control level of 4 and at 30% duty cycle. The sonicated sample was centrifuged at 9,000 x g for 10 min to remove cell debris, then the supernatant (12 ml) was ultracentrifuged at 100,000 x g for 1 h to separate the membrane fraction (1 ml) from the supernatant. The enzyme reactions were carried out at 90℃ for 15 min in a solution (200 ml) containing 1.2 mM 5-bromo-4-

chloro-3-indolyl-β-glucopyranoside (X-Glu) and 5 ml of each fraction, as the enzyme source, in 50 mM phosphate buffer (pH 6) with 0.3 M NaCl. After the reaction, the solution was cooled in ice and diluted with 1 ml of water; the absorbance at 620 nm was immediately measured. As a control, the assay reactions were performed under the same conditions but without X-Glu to subtract the turbidity derived from each fractionated sample.

To analyze the solubilizing effect of Triton X-100, suspension I was also heated with and without 2.5% Triton X-100 at 85℃ for 10 min and the supernatant was obtained by centrifugation at 15,000 x g for 10 min. The activity of the supernatants was measured using X-Glu as shown above.

Dependence of the BGPh Activity on Triton X-100 - The enzyme reactions were carried out at 98℃ for 20 min in a solution (200 ml) containing 3 mM p-Nph-β-D-Glcp (a p-nitrophenyl saccharide) and 57.5 pM of the purified His-BGPh in 50 mM phosphate buffer (pH 6) with Triton X-100 and 0.1 M NaCl. The concentration of Triton X-100 in the reaction solution was varied from 0.1% to 0.00002%. The reaction was terminated by the addition of 1 M Na2CO3 (1 ml), then centrifuged at 15,000 x g for 10 min. The concentration of the p-Nph group in the supernatant was quantified by measuring the absorbance at 400 nm.

Measurement of the Kinetic Parameters - The enzyme reactions were carried out at 90℃ in a solution (200 ml) containing the substrate and the purified His-BGPh in 50 mM phosphate buffer (pH 6) with 0.1% Triton X-100 and 0.3 M NaCl. For the hydrolysis of p-nitrophenyl (p-Nph) β

- D-saccharides, the reaction was terminated by the addition of 1 M Na2CO3 (1 ml), then centrifuged at 15,000 x g for 10 min. The concentration of the p-Nph group in the supernatant was quantified by measuring the absorbance at 400 nm. For the hydrolysis of β - D-glucoside, the released glucose was analyzed with a Glucose C-II Test kit (Wako Pure Chemicals, Japan). Initial velocities were obtained directly from the initial slopes of the time course plots. The Km and kcat values were calculated using the Michaelis-Menten equation and the least squares method (12). The subsite affinity for a long alkyl chain was determined using the method reported previously (13-15) on the basis of the subsite theory (16).

Optimum Temperature and Optimum pH - The optimum temperature was measured as follows: the assay mixture (200 ml), which contained 3 mM p-nitrophenyl β - D-glucopyranoside (p-Nph-β - D-Glcp) in 150 mM citrate buffer (pH 5.0) and 1 ml of suspension I (BL21(DE3)/pET-11a/BGPh), was heated at 85℃ for 10 min. The enzyme reactions were carried out in duplicate at temperatures ranging from 50 ℃ to 100 ℃ for 30 min. Optical density measurements at A405 were performed as described for the enzyme assays.

To determine the optimum pH, the assay mixture (200 ml), which contained 1 ml of heated suspension I (BL21(DE3)/pET-11a/BGPh or BL21(DE3)/pET-15b/BGPh) and p-Nph-β - D-Glcp (3 mM) in 139 mM buffer systems, was heated at 90 ℃ for 30 min. The pH of the reaction mixtures ranged from 3.9 to 5.5 in sodium acetate buffer and from 5.5 to 7.99 in phosphate buffer. Optical density measurements

at A405 were performed as described for the enzyme assays.

Thermostability - The His-BGPh solutions (29 nM) in 50 mM phosphate buffer (pH 6.0) containing 100 mM NaCl and 0.1% Triton X-100 were heated in sealed Eppendorf tubes at 90℃ in various increments up to 24 h. The heated enzymes were assayed in duplicate in phosphate buffer (pH 6.0) at 90℃ for 20 min as described for the determination of optimum temperature.

Sequence Alignment, Phylogenetic Tree, and Hydropacy Profile - Sequence alignment of β - glycosidases was performed using the GeneWorks program (IntelliGenetics, Inc.) based on a PAM-250 scoring matrix. The enzymes of interest were: β - glycosidase (BGPh) studied in this paper and β - mannosidase (BMPh) from P. horikoshii (8, 9), β - glucosidase (BGPf) and β - mannosidase (BMPf) from P. furiosus (17), and β - glycosidase (Sβ - gly) fromSulfolobus solfataricus (18). Phylogenetic trees for the same sequences were constructed using the GeneWorks program based on the unweighted pair group method with an arithmetic mean (19). Each hydropacy profile was analyzed with DNASIS-Mac v2.0 software based on the Kyte and Doolittle method (20).

RESULTS AND DISCUSSION

Localization of the Activity in E. coli Membrane - The intracellular localization of His-BGPhwas examined (Table I).

Table I. Cellular localization of the activity. The transformant *E. coli* BL21(DE3)/pET15b/BGPh cells were used for this experiment. The enzyme reaction were performed at 90°C and pH 6 for 15 min using X-Glu as substrate, and then A_{620} was measured as shown in "MATERIALS AND METHODS".

Cell fractions	Activity after each treatment (A_{620})				
	Sonication	Non-heated	Heated	Non-heated with 2.5% Triton X-100	Heated with 2.5% Triton X-100
Suspension I	0.585	0.585	0.567	0.485	0.428
Supernatant at 9,000 xg	0.112	ND	ND	ND	ND
Supernatant at 15,000 xg	ND	0.008	0.005	0.107	0.255
Supernatant at 100,000 xg	0.010	ND	ND	ND	ND
Fraction precipitated at 100,000 xg	0.478	ND	ND	ND	ND

ND; not determined.

The induced cells were disrupted by sonication and centrifuged to separate the cell components. The membrane fraction was precipitated by ultracentrifugation at 100,000 x g from the supernatant recovered by centrifugation at 9,000 x g. The activity was present in the membrane fraction whereas no activity was detected in the soluble fraction after the ultracentrifugation. His-BGPhwas solubilized from the cell suspension (suspension I) by heating with a detergent, Triton X-100; the enzyme was not solubilized by heating without Triton X-100. The solubilizing efficiency with Triton X-100 was elevated by heating up to 85℃ , whereas only 22% of the activity was extracted at room temperature. The best condition for the solubilization was 2.5% Triton X-100 at 85℃ for 15 min. The native-type BGPhwas also solubilized under the same condition as His-BGPh (data not shown); however, the denaturation with 8 M urea and the renaturation by direct dilution with buffer had no effect on the solubilization of the activity (data not shown). These

facts strongly indicate that BGPh is a thermostable membrane protein solubilized by Triton X-100.

His-BGPh was purified by one-step affinity chromatography using Ni-conjugated Sepharose. Since the recovery of the active enzyme was decreased to a few percent by the elimination of Triton X-100 from the chromatographic washing and elution buffers, the presence of Triton X-100 in the buffer system was essential for the stabilization of BGPh.

As shown in Figure 1, the activity of BGPh was dependent on the concentration of Triton X-100. At 0.00002% Triton X-100, the activity decreased to 10% of that with 0.1% Triton X-100. Furthermore, BGPh was stabilized in the presence of 0.1% Triton X-100: the half-life of the activity was 15 h at 90℃ and pH 6.0 (Fig. 2). These facts also suggest that BGPh is the membrane protein.

The Substrate Specificity of BGPh - For BGPh both with or without His-tag, the optimum pH was 6.0 (Fig. 3) and the optimum temperature was over 100 ℃ (Fig. 4). The substrate specificity of His-BGPh was examined using p-Nph-β-D-saccharides and β-D-glucosides as substrates. The specificity is summarized in Table II in comparison with that of Sβ-gly (7, 21).

Table II. Comparison of the kinetic parameters between his-tagged BG*Ph* from *P. horikoshii* and Sβ-gly from *S. solfataricus* strain MT-4 against *p*-Nph-β-D-saccharides and β-D-glucosides.

Substrates	His-BG*Ph* (90°C and pH 6.0)			Sβ-gly[a] (75°C and pH 6.5)		
	k_{cat} (sec^{-1})	K_m (mM)	k_{cat}/K_m ($mM^{-1}sec^{-1}$)	k_{cat} (sec^{-1})	K_m (mM)	k_{cat}/K_m ($mM^{-1}sec^{-1}$)
Laminaribiose	184	138.23	1.33	908	1.0	908.0
Cellobiose	194	1698.18	0.11	1333	30.0	44.4
Cellotriose	ND[b]	ND	ND	197	3.0	66
Cellotetraose	ND	ND	ND	584	1.7	343
β-Gentiobiose	ND	ND	ND	1360	100	14
p-Nph-β-D-Glc*p*	79	0.35	225.67	542	0.5	1084.0
p-Nph-β-D-Gal*p*	123	1.30	94.34	1020	4.7	217.0
p-Nph-β-D-Xyl*p*	3	0.10	31.83	284	4.0	71.0
p-Nph-β-D-Man*p*	2	0.14	14.60	NH[c]	NH	NH
Salicin	44	1.96	22.20	880	5.0	175.9
Methyl-β-D-Glc*p* (Alkyl : C_1)	35	40.74	0.85	-[d]	-	-
n-Amyl-β-D-Glc*p* (Alkyl : C_5)	31	2.02	15.11	256	1.1	232
n-Hexyl-β-D-Glc*p* (Alkyl : C_6)	33	0.54	60.28	263	1.0	263
n-Octyl-β-D-Glc*p* (Alkyl : C_8)	34	0.20	170.70	313	0.7	434
n-Nonyl-β-D-Glc*p* (Alkyl : C_9)	39	0.08	471.57	-	-	-
n-Decyl-β-D-Glc*p* (Alkyl : C_{10})	37	0.08	469.62	-	-	-
n-Undecyl-β-D-Glc*p* (Alkyl:C_{11})	43	0.05	944.37	-	-	-
n-Dodecyl-β-D-Glc*p* (Alkyl:C_{12})	36	0.03	1152.90	-	-	-

[a] Citated from references (7, 21).

[b] ND; The parameters were not determined because of too high K_m values.

[c] NH; The substrate was not hydrolyzed by Sβ-gly.

[d] -; The parameters were not reported in the references.

His-BGPh hydrolyzed aryl glycosides efficiently, showing kcat/Km values decreasing in the order p-Nph-β-D-Glcp > p-Nph-β-D-Galp > p-Nph-β-D-Xylp > p-Nph-β-D-Manp. Beta-linked glucose dimers tested were poorly hydrolyzed; the order of preference was β 1-3 > β 1-4 > β 1-6. The kcat values of BGPh without His-tag for these β-linked glucose dimers approached 400 sec^{-1}, which is comparable with those of Sβ-gly (Table II). His-BGPh probably had approximately 50% of the activity of BGPh due to interference by the His-tag located at the N-terminus, (Fig. 3). Surprisingly, the best substrates for His-BGPh were β-D-glucosides with long alkyl chains (LA-β-D-Glcp). The Km values decreased according to the elongation of the alkyl chain from C_1 to C_{12}, although the kcat value was constant (approximately 35 sec^{-1}) for each alkyl-β-D-Glcp. The kcat values of native type BGPh for LA-β-D-Glcp approached 70 sec^{-1}, calculated on the basis of the value of His-BGPh, estimating a 50% decrease in the activity from the inhibitory effect of the His-tag. The value was also appreciable, around 30% of that of Sβ-gly (Table II). The Km value of His-BGPh for the hydrolysis of n-Dodecyl-β-D-Glcp (alkyl chain : C_{12}) was extremely low, 30 mM at 90℃ and pH 6.0. Of the substrates examined thus far, the best substrate was n-Dodecyl-β-D-Glcp as shown in Table II. The kcat/Km value of His-BGPh against n-Dodecyl-β-D-Glcp was 5 times higher than that of p-Nph-β-D-Glcp and 870 times higher than that of laminaribiose. Even the value for n-Octyl-β-D-Glcp was 0.76 times higher than that of p-Nph-β-D-Glcp and 128 times higher than that of

laminaribiose. The kcat/Km value of Sβ - gly against n-Octyl-β - D-Glcp, with the longest alkyl chain so far examined (21), was 0.4-fold higher than that for p-Nph-β - D-Glcp and 0.48-fold higher than that for laminaribiose. Laminaribiose and cellobiose were not good substrates for the hydrolysis of His-BGPh because of their Km values higher than 100 mM. His-BGPh also hydrolyzed cellotriose and cellotetraose with low efficiency: the kinetic parameters were not determined because of the extremely high Km value, whereas Sβ - gly was able to hydrolyze these oligosaccharides with high efficiency: the kcat/Km values descended in the order; cellotetraose > cellotriose > cellobiose. Thus, the substrate specificity of His-BGPh is different from those of the other β - glycosidases, including Sβ - gly (7, 17, 21-23). BGPh has a novel substrate specificity with high efficiency to hydrolyze LA-β - D-Glcp and low efficiency to hydrolyze any β - linked glucose dimer which is more hydrophilic than aryl- or alkyl-β - D-Glcp. The subsite affinity ($A_{(C11)}$) to bind a long alkyl chain $_{(C11)}$ was calculated according to the following equation; $A_{(C11)}=RT((kcat/Km)_{\text{for n-Dodecyl-}\beta\text{-D-Glcp}} / (kcat/Km)_{\text{for Methyl-}\beta\text{-D-Glcp}})$. The affinity was determined to be 4.26 kcal/mol. The value was reasonable when compared with the highest affinity (4.23 kcal/mol) known, that of the recognition of one glucose unit in the subsite structure of Saccharomycopsis amylase (13, 14). These facts indicate that the hydrophobicity of the aglycon part of the substrates is strongly recognized by the BGPh molecule and the hydrophobic substrates, including aryl- and LA-

β - D-Glcp, are hydrolyzed effectively with low Km values due to hydrophobic interaction between the aglycon moiety and the BGPh molecule. Thus, BGPh might be useful to synthesize novel β - glycosides, including new biosurfactants, using its transglycosylation activity because of its stability in organic solvents (data not shown).

Henrissat proposed an alternate and complementary classification scheme for glycosyl hydrolases based on amino acid sequence similarities (24-26). For example, glycosyl hydrolase family 1 is composed of exo-acting, β - specific enzymes with similar amino acid sequences. The five β - glycosidases, including BGPh from the archaea domain (as shown in Fig. 5), belong to family 1. Some family 1 glycosyl hydrolases also have glycosyl transferase activities. The S. solfataricus β - glucosidase has been implicated in the glycosylation of membrane lipid components (27). Similarly, the enzymatic analysis of BMPf predicted its possible role in the synthesize of intracellular components including protein, membrane components, or other compounds (17). Since the localization of BGPh on E. coli membrane strongly indicates the intimate interaction of the enzyme and lipid components, the detection of BGPh on the Pyrococcus cell surface using antibody against the enzyme must be done to clarify its true function in the Pyrococcus cell.

The Structural Elements Responsible for Membrane Localization and the Conservation of Residues Forming the Active Site - The sequence alignment among BGPh and four

different β - glycosidases, whose biochemical characteristics have been reported (7, 17, 21-23), is shown in Figure 5. According to the phylogenetic analysis based on the alignment, the tree has three branches : one corresponding to a β - glycosidase group that includes BGPf and Sβ - gly; another containing BMPh and BMPf, which were close to β - mannosidase. BGPh belongs to the third branch, located some distance from the first two branches. The polypeptide length of BGPh is also approximately 13% shorter than those of the other four β - glycosidases and might be one of the shortest sequences so far reported (8, 17, 18, 28). As shown in Figure 5, the residues E155 and H111 of BGPh correspond to E206 and H150 as the putative acid/base catalyst in the Sβ - gly molecule (28, 29), whose steric structure has been reported (30). The residues E324 and R75 of BGPh correspond to E387, the nucleophile, and R79 in the spatial proximity of the nucleophile (28, 29). The complex structure of Bacillus polymixa β - glycosidase with the inhibitor gluconate has been reported (31). The BGPh residues, Q19, H111, N154, E155, Y267, E324, W362, E369, and W370 are completely conserved (Fig. 5) and correspond to the B. polymixa β - glycosidase residues, Q20, H121, N165, E166, Y296, E352, W398, E405, and W406, which form the intimate interaction with the inhibitor (31).

To understand the localization mechanism of BGPh to the membrane, a major structural difference between BGPh and the other soluble β - glycosidases was analyzed using the sequence alignment and the steric structure of Sβ -

gly (30). The Sβ - gly molecule has the classic $(\beta\alpha)_8$ barrel fold first seen in the structure of triose phosphate isomerase (32). For BGPh, the prominent deletion of more than 30 residues was found after the 78th residue, as indicated in Figure 5. The deletion region of BGPh corresponds to loops from the 89th to 125th residues of Sβ - gly, mainly shielding the helices 3 and 4 from solvent. The hydrophilic loops, which pack against the outer face of the barrel helices 3 and 4, were not present in the BGPh molecule. A tetrameric Sβ - gly structure has been reported, in which these loop regions were located at the four edges of regular tetragonal molecular arrangement (30). Figure 6 illustrates the location of the four hydrophilic edges and four hydrophobic areas which appear following the removal of the hydrophilic loops. Since BGPh as well as Sβ - gly was proved to be tetramer by gel filtration using buffer containing 0.01% Triton X-100 (data not shown), the deletion of these hydrophilic loops probably results in the exposure of helices 3 and 4 to the solvent at the four edges of the tetrameric structure. The exposed hydrophobic areas might interact with lipid components to embed the molecule in the membrane.

The increased hydrophobicity at barrel helices 3 and 4 is also indicated by the comparison of the hydropacy plots of BGPh and Sβ - gly, as shown in Figure 7. Two major hydrophobic clusters were observed in the region of BGPh between residues 79 and 210 corresponding to the region of Sβ - gly between residues 90 and 265. These residues form tertiary structures from the end of β - sheet 2 to the

beginning of β - sheet 5 of the $(\beta\alpha)_8$ barrel fold (30). The first cluster was located between residues 79 to 114, forming a helix with a loop shortened by the deletion, α-helix 2, and β - sheet 3. The second cluster was present between residues 131 and 210, corresponding the barrel fold between a-helices 3 and 4 exposed to solvent. A hydrophilic module that might be important for enzyme orientation on the membrane was found between residues 114 to 131, corresponding to the hydrophilic helices at the molecule surface located between the β - strand and the α-helix in the third repeat of the barrel fold. The two hydrophobic clusters, but not the hydrophilic module, were lacking in the corresponding region of Sβ - gly (18).

A mechanism for the localization of BGPh is proposed here based on the possible hydrophobic interaction between the membrane and the exposed hydrophobic helices 3 and 4 at the four edges of the tetrameric structure exposed by the deletion of the hydrophilic loops. Furthermore, the mechanism is well supported by the hydropacy profile of BGPh, in which the hydrophobic cluster is formed by the barrel fold between a-helices 3 and 4. The exposed hydrophobic areas may lead the hydrophobic substrates to the active site and bind them there. However, further studies using the crystallographic analysis are needed for a more definitive description of the detailed mechanism for recognition of the hydrophobic aglycon part, including a long alkyl-chain.

As described above, the present invention provides a novel β -glycosidase. The β -glycosidase is stable under

extreme conditions. Therefore, the β-glycosidase can be used to develop heterosaccharides with high optical purities.

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All publications, patents and patent applications

cited herein are incorporated herein by reference in their entity.

The invention has been described in detail with reference to various embodiments, and it will now be apparent from the foregoing to those skilled in the art that changes and modifications may be made without departing from the invention in its broader aspects, and it is the invention, therefore, in the appended claims to cover all such changes and modifications as fall within the true spirit of the invention.

The following is information on sequences described herein:

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